ROLLING CIRCLE REPLICON EXPRESSION VECTORS

Cross-Reference to Related Applications

This invention is a continuation-in part application of U.S. patent application Ser. No. 09/505,477, filed February 16, 2000.

Field of the Invention

This invention relates to the field of single stranded circular DNA (ssDNA) viruses that infect eukaryotic hosts. In particular this invention relates to viral vectors having utility in vaccine therapy by expressing a heterologous peptide or polypeptide, which is capable of eliciting an immunization reaction in an eukaryotic host. Among the viruses in this field are the Geminiviruses, Nanoviruses, and Circoviruses.

Background of the Invention

The circular single stranded DNA (ssDNA) viruses that infect eukaryotic hosts belong to several different virus taxonomic families (Van Regenmortel *et al.*, 1999; Pringle, 1999). Circoviruses, Circinoviruses (Mushahwar *et al.*, 1999), Gyroviruses and Parvoviruses infect vertebrates; some Parvoviruses (subfamily *Densovirinae*) also infect invertebrate hosts while Geminiviruses and viruses in the genus *Nanovirus* infect plants. There is recent evidence that the viruses currently classified as Circoviruses evolved from Nanoviruses and have switched from a plant to a vertebrate host (Gibbs and Weiller, 1999).

Geminiviruses, Nanoviruses, and Circoviruses are all small circular ssDNA viruses that appear to be fairly closely related, in that they use the same basic rolling-circle mechanism of replication (RCR) and employ very similar life cycle strategies. Recently published data indicate that some plant RCR viruses—dicot-infecting begomoviruses and at least one Nanovirus genomic component even co-exist in some plant infections, with the geminiviral component of the infection presumably providing movement and propagation functions for the Nanovirus element, which functions as a sort of autonomously replicating satellite virus (Mansoor *et al.*, 1999; Saunders and Stanley, 1999). The genomes of all of the plant RCR viruses, and related vertebrate-infecting Circoviruses are small, single-stranded and circular. The

Geminiviruses have mono- or bi-partite genomes, with each genomic component between 2.5 and 3.0-kb. The Nanoviruses have multipartite genomes, generally with at least six, and up to ten, circular subgenomic ssDNAs, each of about 1.0-kb (Katul *et al.*, 1998; Boevink *et al.*, 1995; Burns *et al.*, 1995). The Circoviruses PCV and BFDV have circular ssDNA genomes between 1.75- and 2.0-kb that encode at least two proteins. It is hypothesized that the PCV and BFDV genomes evolved after a recombination event between at least two Nanovirus subgenomic component and a vertebrate RNA-infecting virus which contributed a small portion of the new virus's replication associated protein.

The life cycle of the plant RCR viruses and Circoviruses consists of the following stages, (reviewed by Palmer and Rybicki, 1998; Hanley-Bowdoin *et al.*, 1999):

1. Entry of the ssDNA of the virus into the cytoplasm of the host cell as virion or ssDNA-protein complex.
2. Entry of the ssDNA into the host cell nucleus. This could be a passive process, or may be mediated by the viral capsid protein and/or movement proteins (Lazarowitz, 1999)
3. Conversion of the ssDNA genome into dsDNA presumably mediated by the host DNA repair system. This conversion of the virion DNA into circular dsDNA is required for replication of all RCR replicons, as the "replicative form" (RF) dsDNA intermediate is the template for transcription of the viral genome and therefore expression of viral proteins. The RF DNA becomes associated with host histone proteins and exists as a minichromosome-like structure in the nucleus of infected cells (Abouzid *et al.*, 1988).
4. Transcription of "early" genes—those required for viral replication—by the host RNA polymerase II complex. Production of the viral replication-associated protein (Rep) then results in initiation of RCR of the RF DNA.
5. When the viral RF form reaches a certain critical concentration level in the host cell nucleus, viral transcription regulatory proteins down-regulate transcription of early genes, and stimulate transcription of the viral "late" genes, including the structural protein(s) and proteins required for dissemination of the viral genome.

6. The "late" viral proteins sequester ssDNA produced during replication, move it out of the cell nucleus and ultimately out of the infected cell, either as a ssDNA-protein complex, or as assembled virions.

The plant RCR viruses and their relatives the Circoviruses all encode a replication-associated protein (Rep) that is absolutely required for replication of the virus genomic components (Mankertz *et al.*, 1998; Elmer *et al.*, 1988; Hafner *et al.*, 1997). All other proteins are dispensable for replication, and may be involved in such functions as: movement from cell-to-cell; encapsidation of the virus genome; shuttling of the virus genome between the nucleus and the cytoplasm of infected cells; transcriptional activation or repression of genes in the host or viral genome. The Rep proteins of these RCR viruses bear some distant relationship to replication initiator proteins of some ssDNA plasmids, as well as of members of the *Microviridae*, such as coliphage phiX174 (Ilyina and Koonin, 1992), and has led to speculation that the plant RCR viruses and Circoviruses evolved from prokaryotic ssDNA replicons. The Rep proteins of all of these replicons is a sequence specific DNA binding protein with site specific cleavage and joining activity. In all cases, Rep, probably in association with host enzymes and possibly other viral proteins (Castellano *et al.,* 1999) binds RF DNA at specific sequences and nicks the plus strand at a specific point. In the plant RCR viruses and Circoviruses this specific point occurs within a conserved nonanucleotide sequence that occurs in the loop of a stem-loop structure in the viral intergenic region. The sequence of this nonanucleotide sequence is well conserved between all RCR viruses of plants and Circoviruses: in Geminiviruses the sequence of the nonanucleotide origin of RCR is: TAATATTAC (Palmer and Rybicki, 1998; Hanley-Bowdoin *et al.*, 1999); in Nanoviruses and Circoviruses the sequence is TANTATTAC (Meehan *et al.*, 1997; Hamel *et al.*, 1998; Morozov *et al.*, 1998). Thus, the consensus sequence for nonanucleotide origin of replication for these viruses is TANTATTAC. The Rep protein-mediated cleavage of this nonanucleotide sequence occurs between positions 7 and 8. The minimum amount of sequences that are required to be present on a DNA molecule so that it can be replicated in a reaction mediated by an RCR virus Rep protein are referred to as the RCR virus's minimal origin of replication (minimal ori). The minimal origin of replication is empirically determined, and virus species-specific; the term "minimal ori" is used interchangeably with "ori", and "origin of replication". In general, the minimal ori includes: (1) the

viral stem-loop structure with TANTATTAC nonanucleotide sequence present in the loop; (2) generally, at least 90 base pairs 5' to the start of the stem-loop structure and (3) generally, at least 10, but in many cases up to 100 bases, 3' of the end of the stem-loop structure. The minimal ori is always contained within the main viral intergenic region. The main viral intergenic region (IR) is a non-coding DNA sequence that contains the stem-loop structure, TANTATTAC sequence, binding sites for the Rep protein, the minimal ori, and promoter sequences for driving transcription of viral genes in both orientations relative to the IR. In Geminiviruses of genus *Begomovirus*, the minimal ori is contained within the common region, a sequence within the IR that is common to both DNA A and DNA B genetic components since the sequence is required to be present in *cis* for replication of both components. Likewise, the minimal *ori* of *Nanoviruses* is contained within the viral common region, present on all genome components. In *Curtoviruses*, the minimal *ori* is contained within the IR, and *Mastreviruses* the minimal *ori* is within the Long IR, but sequences in the Short IR are also required for replication. In *Circoviruses* the minimal ori is contained within the IR, and constitutes the stem-loop structure, TANTATTAC sequence and sequences flanking the stem-loop structure (Mankertz *et al.*, 1997).

Replication of the plant RCR viruses and Circoviruses is entirely dependent upon a single virally-encoded replication initiator protein (Rep). Rep proteins of these viruses all contain three conserved protein motifs which are also present in replication intiator proteins from prokaryotic RCR replicons (Ilyina and Koonin, 1992; Palmer and Rybicki, 1998; Mankertz *et al.*, 1998; Meehan *et al.*, 1997; Bassami *et al.*, 1998; Gibbs and Weiller, 1999). The function of motif I (FTLNN in Circoviruses, FTLNY in Nanoviruses and FLTYP in Geminiviruses), is unknown; Motif II (GXXXHLQGF in Circoviruses, GXXHLQGF in Nanoviruses and GXXHLH(A/V)L in Geminiviruses) is probably involved in metal ion coordination. Motif III [(V/N)(R/K)XYXXK in all three groups] contains a conserved tyrosine residue that participates in phosphodiester bond cleavage and in the covalent linkage of Rep to the 5' terminus of the nicked nonanucleotide motif at the origin of replication. The Rep proteins of these three groups of viruses also contains a fourth conserved motif, a nucleotide triphosphate-binding domain ($GX_4GKXXWARX_{28-29}DD$) that may indicate that these proteins possess helicase activity.

Apart from their functions in RCR, Rep proteins and ancillary replication-associated "early" gene products also seem to have transcription factor activity, and are capable of controlling viral and perhaps also host gene expression. Geminivirus Rep proteins can interact with both mammalian and plant Retinoblastoma protein (Rb) homologues (Xie *et al.*, 1995; 1996; Grafi *et al.*, 1996; Xie *et al.,* 1996; Ach *et al.,* 1997). Rb belongs to a protein family that controls cell cycle progression by sequestering transcription factors necessary for entry of the cell cycle into S phase. There is also evidence that infection of plants with Geminiviruses such as tomato golden mosaic begomovirus (TGMV) is associated with an increase in the levels of proliferating cell nuclear antigen (PCNA), a DNA polymerase processivity factor required in cellular DNA replication (Nagar *et al.*, 1995). These viruses thus appear to possess the ability to modify the host environment to one that allows viral DNA replication. At present, the exact mechanisms by which these viruses modify the host cell cycle are unclear. This could be achieved exclusively through interaction of viral proteins (such as Rep) with host proteins (such as Rb). It is also possible that transcriptional activation or repression of host genes mediated by the transcription factor activity of viral protein(s) may also be involved in resetting the cellular environment to one that is permissive for viral replication.

Of this group of closely related RCR viruses, only Geminiviruses have been exploited as gene vectors in plant cells. Recombinant viral vectors that have a foreign gene inserted in place of a begomovirus coat protein can sometimes infect permissive dicotyledonous plant hosts and move systemically in infected plants (Ward *et al.*, 1988; Hayes *et al.*, 1988; Sudharsha *et al.*, 1998). Vectors that contain part of the begomoviral genome, including at least three open reading frames (AC1 [=Rep], AC2 and AC3) driven by their own promoters, and containing the viral origin of replication, can replicate in transfected dicotyledonous plant cells (Palmer *et al.*, 1997). Mastrevirus-derived vectors that contain the two genes (Rep and RepA) necessary for replication of the viral genome and expression of the viral late genes, together with the viral origins of replication, can replicate in cells derived from monocotyledonous cereal plants (Palmer *et al.*, 1997; Palmer *et al.*, 1999).

Summary of the Invention

One aspect of this invention is a polynucleotide capable of rolling circle replication in an eukaryotic host. One aspect of this invention is a rolling circle DNA replicon (RCR replicon) which replicates in a host eukaryotic cell. Another aspect of the invention is a RCR replicon which has a truncated replication cycle. Another aspect of the invention is a polynucleotide or a RCR replicon which has the following elements, present on the same DNA molecule: A Rep gene open reading frame (ORF) from a virus belonging to the viral taxonomic families *Geminiviridae*, *Circoviridae* or genus *Nanovirus*, said Rep gene open reading frame is placed under transcriptional control of a promoter, which promoter is placed 5' of the gene; any sequences that are required to be present in *cis* on the rolling circle DNA replicon in order that the Rep protein might promote replication of the rolling circle DNA replicon; an expression cassette for expression of an ancillary protein that is capable of creating a cellular environment permissive for replication of the rolling circle DNA replicon in the host cell of interest; and at least one expression cassette with an RNA polymerase II promoter, a multiple cloning site, and transcription termination and polyadenylation signals suitable for transcription of RNA molecules not normally intrinsic to a geminiviral, circoviral or nanoviral genome.

Another aspect of the invention is a RCR replicon, which replicates in a host eukaryotic cell, and which has a promoter that can function in a host eukaryotic cell type of interest.

Another aspect of the invention is a RCR replicon, which replicates in a host eukaryotic cell, and which has a promoter that has some tissue- or cell-type specificity.

Another aspect of the invention is a RCR replicon for a host cell, which has a promoter that is inducible by chemical or other environmental induction.

Another aspect of the invention is a RCR replicon which replicates in a host eukaryotic cell, and which has sequences that are required to be present in *cis* on the rolling circle DNA replicon in order that the Rep protein might promote replication of the rolling circle DNA replicon, said sequences derived from the group consisting of Nanoviruses, Circoviruses, begomoviruses and curtoviruses.

Another aspect of the invention is an RCR replicon which replicates in a host eukaryotic cell, and which has sequences that are required to be present in *cis* on the

rolling circle DNA replicon in order that Rep might promote the replication of the rolling circle DNA replicon. These sequences are:

(a) the origin of replication from the same virus from which the Rep protein gene was derived; said origin of replication containing the conserved stem-loop structure;

(b) a TANTATTAC sequence, where "N" may be A or C or G or T;

(c) sufficient stem-loop structure flanking sequences to provide the minimal origin of replication for the virus.

Another aspect of the invention is an RCR replicon derived from a Mastrevirus which replicates in a host eukaryotic cell, and which has sequences that are required to be present in *cis* on the rolling circle DNA replicon in order that Rep might promote the replication of the rolling circle DNA replicon. These sequences are:

(a) the origin of replication from the same virus from which the Rep protein gene was derived; said origin of replication containing the conserved stem-loop structure;

(b) a TANTATTAC sequence, where "N" may be A or C or G or T;

(c) sufficient stem-loop structure flanking sequences to provide the minimal origin of replication for the virus;

(d) the Short intergenic region (SIR) derived from the same Mastrevirus that provided the Rep protein gene.

Another aspect of the invention is a RCR replicon which replicates in a host eukaryotic cell, and which has an expression cassette that: (a) functions in expression of an ancillary protein, and (b) which is redundant with the Rep gene expression cassette.

Another aspect of the invention is a RCR replicon which replicates in a host eukaryotic cell, and which has an expression cassette for expression of an ancillary protein and an expression cassette driving the expression of a Rep ORF which expression cassette is from a different virus species from the group of Geminiviruses, Circoviruses and Nanoviruses.

Another aspect of the invention is a method of making a rolling circle DNA replicon which replicates in a host eukaryotic cell, comprising combining:

(a) a Rep gene ORF from a virus belonging to the viral taxonomic

families *Geminiviridae*, *Circoviridae* or genus *Nanovirus*, said Rep gene open reading frame is placed under transcriptional control of a promoter, which promoter is placed 5' of the gene;

(b) any sequences that are required to be present in *cis* on the rolling circle DNA replicon in order that the Rep protein might promote replication of the rolling circle DNA replicon;

(c) an expression cassette for expression of an ancillary protein that is capable of creating a cellular environment permissive for replication of the rolling circle DNA replicon in the host cell of interest; and

(d) at least one expression cassette with an RNA polymerase II promoter, a multiple cloning site, and transcription termination and polyadenylation signals suitable for transcription of RNA molecules not normally intrinsic to a geminiviral, circoviral or nanoviral genome.

Another aspect of the invention is a method of making a rolling circle DNA replicon which replicates in a host eukaryotic cell which replicon has a truncated replication cycle, comprising combining:

(a) a Rep gene open reading frame from a virus belonging to the viral taxonomic families *Geminiviridae*, *Circoviridae* or genus *Nanovirus*, said Rep gene open reading frame is placed under transcriptional control of a promoter, which promoter is placed 5' of the gene;

(b) any sequences that are required to be present in *cis* on the rolling circle DNA replicon in order that the Rep protein might promote replication of the rolling circle DNA replicon;

(c) an expression cassette for expression of an ancillary protein that is capable of creating a cellular environment permissive for replication of the rolling circle DNA replicon in the host cell of interest; and

(d) at least one expression cassette with an RNA polymerase II promoter, a multiple cloning site, and transcription termination and polyadenylation signals suitable for transcription of RNA molecules not normally intrinsic to a geminiviral, circoviral or nanoviral genome.

Another aspect of the invention is a method of discovering the function of a gene or gene segment in a host eukaryotic cell, the method comprising:

(a) inserting a gene or gene segment into the multiple cloning site of the above-mentioned expression cassette in the RCR vector, such that the RNA II polymerase promoter may promote the transcription of the inserted gene or gene segment;

(b) inserting the rolling circle DNA replicon into a host eukaryotic cell; and

(c) discovering a biochemical or phenotypic change in the host eukaryotic cell.

Another aspect of this invention is a polynucleotide comprising elements of a viral genome which is capable of rolling circle replication, whereby the expression of a polypeptide or peptide encoded by the polynucleotide is capable of eliciting an immunization reaction in a host eukaryote. Another aspect of this invention is a polynucleotide comprising elements of a viral genome which is capable of rolling circle replication, whereby the immunization reaction to the expression of a polypeptide or peptide encoded by the polynucleotide in the host eukaryote is potentiated by an inserted nucleic acid sequence encoding an ancillary immunogenic protein. Another aspect of this invention is a polynucleotide comprising elements of a viral genome which is capable of rolling circle replication, whereby the polynucleotide is targeted to a host eukaryotic cell nucleus, which may allow increased expression of a peptide or polypeptide encoded by the polynucleotide which is capable of eliciting an immunization reaction in a host eukaryote.

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Another aspect of the invention is a method of making a polynucleotide comprising elements of a viral genome which is capable of rolling circle replication, whereby the immunization reaction to the expression of a polypeptide or peptide encoded by the polynucleotide in the host eukaryote is potentiated by an inserted nucleic acid sequence encoding an ancillary immunogenic protein.

Another aspect of the invention is a method of making a polynucleotide comprising elements of a viral genome which is capable of rolling circle replication, whereby the polynucleotide is targeted to a host eukaryotic cell nucleus, which may allow increased expression of a peptide or polypeptide encoded by the polynucleotide which is capable of eliciting an immunization reaction in a host eukaryote.

Another aspect of the invention is a method of immunizing a eukaryotic host, whereby a polynucleotide comprising elements of a viral genome which is capable of rolling circle replication is administered to a eukaryotic host, whereby said polynucleotide is capable of expression of an inserted nucleic acid sequence, the expression of said nucleic acid sequence capable of eliciting an immunization in a eukaryotic host.

Brief Description of the Drawings

FIG. 1 shows a restriction and genetic map of Construct 1. The PCV Rep Promoter, the PCV Rep Gene with restriction sites, the PCV Ori and cloning vector are shown.

FIG. 2 shows a restriction and genetic map of Construct 2 with pCMV, pCV Rep gene, PCV Ori, and SV40 terminator with G418/kanamycin resistance gene.

FIG. 3 shows the restriction map and genetic map of Construct 6 (p TracerSV40 from Invitrogen Corp). Construct 6 is the backbone of Construct 7. It contains the same GFP-zeocin expression cassette that is present in Construct 7. The NotI – NsiI fragment from Construct 1 was excised and inserted into Construct 6, replacing the SV40 promoter with the PCV fragment to generate Construct 7. The pTracer™-SV40 vector is available from Invitrogen Corp. (Carlsbad, California).

FIG. 4 shows a Southern blot of DNA isolated from cells transfected with PCV-containing constructs and control DNAs. Two and a half micrograms of total DNA from transfected cells was digested with an excess of *Dpn*I restriction enzyme and electrophoresed in a 1.0% TAE agarose gel and stained with eithidium bromide. DNA was transferred to a nylon membrane by capilliary transfer. The Southern blot was hybridized with a probe prepared from construct 1, which has homology with all input plasmid DNAs. Lanes 1 to 9 contain DNA isolated from COS-7 cells transfected with the following plasmids: Lanes 1 and 2 (Construct 1, DNA isolated at day 2 and day 4 post-transfection); Lanes 3 and 4 (Construct 2, day 2 and day 4 post-transfection); Lanes 5 and 6 (Construct 4, day 2 and day 4 post-transfection); Lanes 7 and 8 (construct 6, day 2 and day 4 post-transfection); Lanes 9 and 11 contain DNA isolated from untransfected cells; Lane 10 contained a DNA molecular weight marker.

Lanes 12 to 19 contain DNA isolated from CHO-K1 cells transfected with the following plasmids: lanes 12 & 13 (Construct 7, day 2 and day 4 post-transfection); Lanes 14 and 15 (Construct 2, day 2 and day 4 post-transfection); Lanes 16 and 17 (Construct 4, day 2 and day 4 post-transfection); Lanes 18 and 19 (Construct 6, day 2 and day 4 post-transfection); and, Lane 20 contains DNA isolated from PCV-positive cell line PK-15, used as a positive control for DNA hybridization. The hybridizing bands run at a significantly lower position, due to the virus's small size (1.8-kb) relative to the plasmid DNAs (greater than 4.0-kb).

FIG. 5 shows the DNA sequence of Construct 1 (SEQ ID NO:1): 5285 bp. Composition: 1216 A; 1277 C; 1514 G; 1278 T; 0 other. Percentage: 23% A; 24% C; 29% G; 24% T; 0% other. Molecular Weight (kDa): ssDNA: 1636.28; dsDNA: 3258.4.

FIG. 6 shows the DNA sequence of Construct 7 (SEQ ID NO:2): 5650 bp. Composition: 1372 A; 1333 C; 1516 G; 1429 T; 0 other. Percentage: 24% A; 24% C; 27% G; 25% T; 0% other. Molecular Weight (kDa): ssDNA: 1747.85; dsDNA: 3483.2.

FIG. 7 shows the structure of plasmid pCI-PCV1-Luc. The PCV1 StuI fragment, the CMV promoter, the intron, the inserted firefly luciferase gene (F.Luc), and the BglII, NheI and EcoRI restriction sites and their nucleotide positions are shown.

FIG. 8 shows the DNA sequence of pCI-PCV1-Luc: 7460 bp (SEQ ID NO:7).

FIG. 9 show the structure of plasmid pGL3-PCV1-Luc. The PCV1 StuI fragment, the SV40 promoter, the inserted firefly luciferase gene (F.Luc), and the BglII, NheI and EcoRI restriction sites and their nucleotide positions are shown.

FIG. 10A shows firefly luciferase activity in CHO cells transfected with CMV based vectors with and without the PCV1 genome at different times post-transfection. The y-axis depicts firefly luciferase units. The x-axis depicts in number of days post-transfection. The open columns are CHO cells with pCI and the hatched columns are CHO cells with pCI-PCV1-Luc. FIG. 10B shows firefly luciferase activity in CHO cells transfected with SV40 based vectors with and without the PCV1 genome at different times post-transfection. The y-axis depicts firefly luciferase units. The x-axis depicts in number of days post-transfection. The open columns are CHO cells with pGL3 and the hatched columns are CHO cells with pGL3-PCV1-Luc.

FIG. 11A shows the structure of plasmid pCI luciferase or Beta-galactosidase. The ampicillin resistance, ColEI ori fragment, the SV40 terminator sequence, the CMV promoter, the inserted firefly luciferase (F.Luc) or Beta-galactosidase (β-gal) are shown. FIG. 11B shows the structure of the plasmid pCI-PCV1 Beta-galactosidase (β-gal). The PCV1 StuI fragment, the CMV promoter, the ColE1 ori fragment, the SV40 terminator sequence, the CMV promoter, and the inserted Beta-galactosidase (β-gal) are shown.

FIG. 12A and 12B shows the upregulation of IgG antibodies from immunization experiments in mice with pCI or pCI-PCV plasmids inserted with either the F.Luc (FIG. 12A) or β-gal (FIG. 12B) gene. The y-axis depicts the level of antibodies against firefly luciferase or beta-galactosidase detected, averaged from five mice. The x-axis depicts the treatment regimen employed.

FIG. 13A and 13B shows the differential upregulation of IgG isotypes after immunization with pCI or PCI-PCV plasmids inserted with either the F.Luc (FIG. 13A) or β-gal (FIG. 13B) gene in mice. The y-axis depicts levels of antibodies against firefly luciferase or beta-galactosidase detected, pooled and averaged from five mice. The x-axis depicts the treatment regimen employed.

Detailed Description of the Invention

In order to facilitate understanding of the invention, certain terms used throughout are herein defined.

"Ancillary immunogenic protein" means a protein which assists in potentiating an immunization reaction by a peptide or polypeptide. By potentiating, the ancillary immunogenic protein increases the immunization reaction to the peptide or polypeptide by at least 1.5- fold, preferably 2-fold or more.

"BFDV" means beak and feather disease virus.

"PCV" means porcine Circovirus.

"CHO cells" means Chinese Hamster Ovary cells

"COS-7 cells" means *Cercopithecus aethiops* (African Green Monkey) kidney cells, transformed with simian virus 40 (SV40).

"D-MEM" means Dulbecco's Modified Eagle Medium.

"*Dpn*I" is a restriction endonuclease which cuts only *dam*-methylated DNA.

"Buffer EC" means DNA condensation buffer.

"Effectene" is a transfection reagent, sold by Qiagen, Inc. (Valencia, California).

"β-gal" means beta-galactosidase, which is an enzyme derived from bacterial beta-galactosidase gene.

"GFP-zeocin" is a fusion gene made by combining the genes for green fluorescent protein and zeocin.

"GM-CSF" means Granulocyte-Macrophage Colony Stimulating Factor. GM-CSF may increase the immunogenicity of antigens by stimulating antibody production mechanisms.

"G418 resistance gene" is a selectable marker gene.

"Heterologous" means not derived or obtained from the same species.

"Histone H1" means Histone H1 protein, which contains nuclear localization sequences and may assist in condensing nucleic acid molecules and targeting associated molecules to the cell nucleus.

"Histone H2A" means Histone H2A protein, which contains nuclear localization sequences and may assist in condensing nucleic acid molecules and targeting associated molecules to the cell nucleus.

"Histone H2B" means Histone H2B protein, which contains nuclear localization sequences and may assist in condensing nucleic acid molecules and targeting associated molecules to the cell nucleus.

"Histone H3" means Histone H3 protein, which contains nuclear localization sequences and may assist in condensing nucleic acid molecules and targeting associated molecules to the cell nucleus.

"Histone H4" means Histone H4 protein, which contains nuclear localization sequences and may assist in condensing nucleic acid molecules and targeting associated molecules to the cell nucleus.

"HUBEC" cell lines means human brain endothelial cell lines.

"IgG" means Immunoglobulin-G.

"Integrated SV40 Large T antigen-expressing gene": The African Green Monkey Kidney cell line COS-7 contains a chromosomally-integrated SV40 virus that has a gene for the Large T antigen protein which is required for SV40 virus replication. Thus, COS-7 cells contain a chromosomally-integrated SV40 Large T

antigen-expresisng gene that is sufficient for episomal replication of SV40 ori-containing plasmids in this cell line.

"Intergenic sequences": The non-coding DNA sequences, wherein the viral origin of replication is situated, that are located between open reading frames of RCR viruses.

"IL-1 beta" means Interleukin 1-beta, a protein which may increase the immunogenicity of antigens by stimulating antibody production mechanisms.

"Lipofectamine" is a cationic lipid used for transfecting mammalian cells. Life Technologies, Inc supplies Lipofectamine.

"F.Luc" means firefly luciferase. It is an enzyme derived from the firefly luciferase gene.

"nonanucleotide": The sequence TANTATTAC, where "N" may be A or C or G or T. This sequence is contained within the loop of the stem-loop structure present in the origin of replication of all RCR viruses in the group of Geminiviruses, Circoviruses, and Nanoviruses.

"neomycin/G418 resistance gene:" A gene that confers to the G418 antibiotic resistance.

"Nuclear targeting proteins" means proteins which target proteins or associated molecules to the cell nucleus.

"*Nsi*I-*Not*I fragment" is a restriction fragment from Construct 1 that is used to create Construct 7.

"ORF" means Open Reading Frame of a gene.

"Passive episomal replicon inheritance:" Process where a replicon present in the nucleus of a cell is passively inherited by both daughter cells upon cell division; the replicons are not actively sequestered into each daughter cell since they do not contain a classical centromere structure, but are nevertheless inherited due to their high copy number in the original undivided cell.

"PMVC cell lines" means porcine microvascular cell lines.

"PCV genome" means the porcine Circovirus genome.

"pCI" means mammalian expression cloning vector from Promega.

"PCV rep": The replication associated protein gene of porcine Circovirus (PCV).

"PCV RCR plasmid" A plasmid that contains the sequences derived from porcine Circovirus which allow the plasmid to replicate by rolling circle replication in a host cell.

"PK-15 cells": Porcine Kidney cell line PK-15 or PK(15). Cell line derived from kidney epithelial cells of *Sus scrofa*. The PK-15 cell line is persistently infected with Porcine Circovirus, type 1 (PCV).

"pCMV-Script": A mammalian cell expression vector obtained from Stratagene, Inc. (La Jolla, CA).

"pCR®-Blunt II-TOPO® vector": a vector useful for cloning of PCR products sold by Invitrogen Corp. (Carlsbad, California).

"PK-15SwaA and PK-15SwaB" are PCR primers used to amplify the PCV genome.

"pTracer™SV40": a mammalian cell expression vector that contains an expression cassette for expression of a GFP-zeocin resistance gene; obtained from Invitrogen Corporation.

"QIAamp DNA Mini Kit" A DNA extraction kit useful for extraction of total DNA from blood and mammalian cells, sold by Qiagen, Inc (Valencia, CA.)

"Rep" means virally-encoded replication initiator protein.

"Rep gene" means a gene from an RCR virus belonging to the group of viruses from the taxonomic families *Geminiviridae* or *Circoviridae* or from the genus *Nanovirus*, which is essential for viral replication and which possesses a nicking and joining activity specific for the TANTATTAC sequence present in the stem loop sequence in the viral origin of replication and which is able to promote replication of an RCR virus.

"Rep gene ORF" is an open reading frame associated with a Rep gene.

"Rep protein" means replication-associated protein, a plasmid-encoded protein that functions as an activator of replication of that plasmid.

"Replicon" means any DNA sequence or molecule which possesses a replication origin and which is therefore potentially capable of being replicated in a suitable cell.

"RCR replicons" are replicons or polynucleotides that reproduce by the rolling circle DNA replication mechanism.

"Rolling circle DNA replication" is a mechanism for the replication of DNA wherein one strand of a parent dsDNA molecule is nicked, and DNA synthesis proceeds by elongation of the 3'-OH end (with progressive displacement of the 5'-end), the unbroken circular strand acting as the template. The partly replicated intermediate is thus a double-stranded circular DNA with a single-stranded displaced tail.

"RCR" means rolling-circle mechanism of DNA replication.

"Rolling circle DNA replicon" means a replicon that reproduces by the rolling circle DNA replication mechanism.

"Rolling Circle Replicon Expression Vectors" means a vector that reproduces by means of the rolling circle DNA replication method.

"RCR vector" means Rolling Circle Replicon Expression Vectors.

"RCR virus" means Rolling Circle Replicon Expression virus.

"ssDNA viruses" means single stranded circular DNA virus.

"SV40 promoter" means simian virus 40 early promoter. Simian virus 40 is a virus of the genus Polyomavirus. SV 40 was originally isolated from kidney cells of the rhesus monkey, and is common (in latent form) in such cells.

"VLPs" means virus-like particles.

The Invention

This invention provides methods for designing and creating a polynucleotide or a rolling circle DNA replicon for an eukaryotic host with elements from RCR viruses from the viral taxonomic families *Geminiviridae* and *Circoviridae*, and from the genus *Nanovirus* that is as yet unassigned to a taxonomic family. We disclose methods for manipulating the genomes of these viruses so that the RCR replicons described in this invention employ only part of the replication cycle of the virus or viruses from which they were originally derived. The RCR replicons are introduced into eukaryotic host cells as double stranded DNA molecules, and thus the form in which the replicon initially enters the host is not usual for the parental virus that normally infects new host cells in an encapsidated ssDNA form. The viral "late" genes that are involved in sequestration of ssDNA, movement of viral DNA out of the

host cell nucleus and assembly of viral DNA into virions are inactivated or deleted in the RCR replicons of this invention.

The invention provides for a polynucleotide capable of rolling circle replication in an eukaryotic host, said polynucleotide comprising:

(a) a first Rep gene encoding a first Rep protein from a first virus selected from the group of genera of family *Geminiviridae*, genera of family *Circoviridae*, and genus *Nanovirus*, wherein said first Rep gene is capable of being expressed in said eukaryotic host;

(b) sequences that are *cis* on the polynucleotide such that the first Rep protein can bring about rolling circle replication of the polynucleotide; and

(c) an open reading frame encoding a protein of interest capable of being expressed in said eukaryotic host, wherein said protein of interest is heterologous to the first Rep protein.

The polynucleotide may lack one or more genes of said first virus. The polynucleotide may further comprise a second Rep gene encoding a second Rep protein from a second virus selected from the group of genera of family *Geminiviridae*, genera of family *Circoviridae*, and genus *Nanovirus*, wherein said second Rep gene is heterologous to the first Rep gene; such a polynucleotide in the ssDNA form may form the RF form and replicate in an eukaryotic host that the first virus cannot replicate in.

These RCR replicons have the following elements, present on the same DNA molecule or polynucleotide:

1. A Rep gene ORF from a virus belonging to the viral taxonomic families *Geminiviridae*, *Circoviridae* or genus *Nanovirus*. This Rep gene ORF is placed under transcriptional control of a promoter, placed 5' of the gene. This promoter is chosen to be one that can function in a cell type of interest, and may additionally have some tissue, or cell-type specificity, or may be induced by the addition of a chemical or by other some other environmental induction.
2. The sequences that are required to be present in *cis* on the RCR replicon in order that the Rep protein might promote replication of the RCR replicon. For Nanoviruses, Circoviruses, begomoviruses and curtoviruses, this is the viral origin of replication that contains the conserved stem-loop structure,

TANTATTAC nanonucleotide sequence, and flanking intergenic sequences. For mastreviruses, these include the long and short intergenic regions.

3. An expression cassette for expression of an ancillary protein that is capable of creating a cellular environment permissive for replication of the RCR replicon in the host cell of interest. This cassette may be redundant with the Rep gene expression cassette described above, or may be an expression cassette driving the expression of a Rep ORF from a different virus species from the group of Geminiviruses, Circoviruses and Nanoviruses.

4. At least one expression cassette with a RNA polymerase II promoter, a multiple cloning site, and transcription termination and polyadenylation signals suitable for transcription of RNA molecules not normally intrinsic to a geminiviral, circoviral or nanoviral genome.

The invention also provides for a method of constructing a rolling circle DNA replicon or a polynucleotide which replicates in a eukaryotic host, comprising:

combining unto a single polynucleotide:

(a) a Rep gene encoding a Rep protein obtained from a virus selected from the group of genera of family *Geminiviridae*, genera of family *Circoviridae*, and genus *Nanovirus*, wherein a promoter is operatively linked 5' to said Rep gene;

(b) sequences that are *cis* on the polynucleotide such that the Rep protein can bring about rolling circle replication of the polynucleotide;

(c) an expression cassette for expression of an ancillary protein that is capable of creating a cellular environment permissive for replication of the rolling circle DNA replicon in the host cell of interest; and

(d) an open reading frame encoding a protein of interest capable of being expressed in said eukaryotic host, wherein said protein of interest is heterologous to the Rep protein.

The invention also provides for a polynucleotide which replicates in a eukaryotic host, wherein said polynucleotide encodes a peptide or polypeptide which is capable of eliciting an immunization reaction in a eukaryotic host. The immunization reaction may be potentiated by the co-expression of an ancillary immunogenic protein, which increases the immunogenicity of the expressed heterologous protein or peptide. The polynucleotide may also be targeted to the

nucleus of an eukaryotic host cell and/or condensed for transport into the eukaryotic host cell's cytoplasm by coating the polynucleotide with proteins. This may increase the expression of the encoded peptide or polypeptide inserted within the polynucleotide, thereby increasing the immunization reaction within the eukaryotic host.

The invention also provides for a method of constructing a polynucleotide which replicates in a eukaryotic host, wherein said polynucleotide encodes a peptide or polypeptide which is capable of eliciting an immunization reaction in a eukaryotic host. Although any peptide or polypeptide may be utilized, peptides or polypeptides which are capable of eliciting an immunization reaction are preferable for use in conjunction with the present invention. Surface or exposed antigens are examples of a peptide or polypeptide which may be desirable for such applications. Alternatively, full length proteins may also be utilized in immunotherapy applications. Detailed structural and functional information about many proteins of interest are well known; this information may be used by one of ordinary skill in the art so as to provide for immunogens having the desired properties allowing the immunological recognition of a protein of interest.

The peptide or polypeptide which is capable of eliciting an immunization reaction may be native or non-native to the eukaryotic host. Preferably, the peptide or polypeptide is non-native to the eukaryotic host, and expression of said peptide or polypeptide elicits an immunization reaction to the expressed foreign antigen, producing an immune response which protects the eukaryotic host against subsequent exposures to organisms which express said foreign antigen in vivo. Examples of foreign antigens include antigens present on microbial pathogens or other pathogenic organisms. Alternatively, the peptide or polypeptide may be native to the eukaryotic host, wherein the expression of said peptide or polypeptide elicits an immunization reaction to a self-antigen, producing an immune response which protects the eukaryotic host against overexpression or abnormal expression of self-proteins. Examples of self-proteins where an immune response against the self-protein antigen may be desired include autoimmune diseases, such as arthritis, lupus erythremastosus or other disease states where the production of antibodies against self-antigens may be useful in combating the disease.

The immunization reaction may be potentiated by the co-expression of an ancillary protein, which may increase the immunogenicity of the expressed heterologous protein or peptide. A preferred embodiment would be the use of proteins known to stimulate an immunological response to the presence of an antigen. Granulocyte macrophage colony stimulating factor (GM-CSF) and IL 1-beta are examples of proteins known to enhance the immunological response to an antigen when administered in the presence of an antigen. The above is not meant to be limiting to the practice of the present invention, and a practitioner of ordinary skill in the art will recognize that any protein which is capable of stimulating or enhancing the immunological response to an antigen is contemplated within the scope of the present invention. The ancillary protein may be co-expressed with the protein immunogen of interest by inserting a polynucleotide sequence encoding the ancillary protein within the polynucleotide vector comprising the polynucleotide encoding the peptide or polypeptide of interest, nucleic acid elements necessary to express the peptide or polypeptide of interest and elements of a viral genome capable of rolling circle replication.

The polynucleotide may also be condensed and targeted to the nucleus of a eukaryotic host cell by coating the polynucleotide with nuclear localization and polynucleotide condensing proteins. Coating the polynucleotide with nuclear localization proteins may have the effect of targeting the polynucleotide to the nuclear structure, transporting the nucleic acid to the site where transcription of the vector template takes place. This may increase the expression of the encoded peptide or polypeptide inserted within the polynucleotide by lessening the opportunities for enzymatic degradation within the cytoplasm of the cell, thereby increasing the available template for transcription and increasing the immunization reaction within the eukaryotic host. Condensation of polynucleotides prior to immunological presentation may also potentiate an immunization response by efficiently packaging the polynucleotide for entry into the host eukaryote's cell cytoplasm. Examples of proteins which are capable of condensing nucleic acids are the histone proteins H2A, H2B, H3, H4 and H1. Compounds, such as polylysine and other polycations, have also been shown to condense nucleic acids. Other non-mammalian proteins, such as the mu protein of adenovirus, have been used to condense nucleic acid. Other proteins

known to those of ordinary skill in the art which condense or target nucleic acids to the cell nucleus may also be used in conjunction with the present invention.

A preferred embodiment of the invention is the use of Histone H1 for condensing and targeting expressed peptides or polypeptides of the invention to the nucleus. Histone H1 both condenses and targets polynucleotides to the cell nucleus by the presence of nuclear localization signals located within the histone H1 protein sequence. Coating of polynucleotides with histone H1 in amounts which condense the polynucleotide sufficiently such that condensation is observable is desired, whereby one of ordinary skill in the art will appreciate that larger-sized polynucleotides, or larger quantities of polynucleotides, will require more histone H1 for condensation of said polynucleotide. Alternative embodiments of the invention include the use of other dual purpose proteins, such as histone H2A, histone H2B, histone H3 or histone H4. Histones H2A, H2B, H3 and H4, like histone H1, condense polynucleotides and contain nuclear localization signal sequences. In other embodiments, combination of proteins above may be used in condensing and targeting polynucleotides provided for in the present invention. For example, combinations of the different histone proteins may be used. In yet other alternative embodiments, proteins which either condense or target coated polynucleotides may also be used in the practice of the invention. For example, the mu protein of adenovirus or polycations such as polylysine, may be used to condense the polynucleotide for entry into the cell cytoplasm in the absence of any nuclear targeting protein.

The polynucleotide expressing the polypeptide or peptide of interest may be administered to the host eukaryote in a variety of ways. Preferably, the polynucleotide is injected into the host eukaryote for uptake and expression in cells. The polynucleotide may be injected in the presence or absence of an adjuvant capable of increasing the immunization reaction of said host eukaryote. Examples of adjuvants include Freund's adjuvant, Ribi adjuvant system, keyhole limpet hemocyanin, cytokines (IL-2, IL-4, IL-10 and IL-12), GM-CSF, microorganisms (e.g. lactobacillus), preformed immune-stimulating complexes (IsCOMs), block co-polymers, cholera toxin, lipopolysaccharides, aluminum salt adjuvants and nitrocellulose-adsorbed antigens. Other adjuvants known to those of skill in the art may also be utilized in combination with the present invention.

Alternatively, the polynucleotide may be modified to increase the uptake of said polynucleotide into cells. This may include encapsulating said polynucleotide with liposomal agents or other agents which increase the uptake of polynucleotides into host eukaryotic cells.

All genes and open reading frames, encoding proteins, can be expressed by having a promoter operatively linked 5' to the gene or open reading frame (i.e., the promoter is at the 5' end of the gene or open reading frame). The promoter is capable of expressing the gene or open reading frame in the eukaryotic host.

The eukaryotic host may be an eukaryotic cell. The eukaryotic cell may be an animal or plant cell. The animal cell is preferably an insect cell, bird cell, or mammalian cell. The mammalian cell may be a CHO cell, COS-7 cell or African Green Monkey kidney cell. The eukaryotic cell may be part of a cell culture, tissue, tissue culture, or organ. The eukaryotic cell may also be part of a whole organism. The whole organism can be a plant or an animal. The animal is preferably an insect, bird, or mammal.

The polynucleotides and RCR replicons of the present invention can also further comprise a prokaryotic selectable and a prokaryotic origin of replication so that the polynucleotide and RCR replicon can be propagated and amplified in a prokaryotic cell. The polynucleotides and RCR replicons of the present invention can also further comprise an eukaryotic selectable and an eukaryotic origin of replication so that the polynucleotide and RCR replicon can be propagated and amplified in an eukaryotic cell using a non-rolling circle replication mechanism.

Utilities:

RCR replicons (or polynucleotides capable of rolling circle replication) are useful for discovery of the function of genes in eukaryotic hosts. RCR replicons are useful for inducing or enhancing a function or trait in a host eukaryotic cell. RCR replicons are useful for down-regulating a gene in a plant or in mammalian cells and thereby altering or even eliminating the function of that gene.

RCR replicons have several properties that will lead to the development of superior gene expression vector properties. The vector initiates a rapid replication cycle leading to earlier gene expression than standard plasmid vectors. This, coupled with its self-amplifying properties, will lead to sustained expression for longer periods

of time as compared with standard plasmid vectors. These properties, coupled with the amplification of substrates for transcription by host machinery, will lead to greater levels and longer enduring levels of target gene expression as compared to standard plasmid vectors. The amplification of 100-1000 copies of the genome per transfected cell will lead to passive inheritance of the RCR replicon infection into daughter cells. This will lead to the development of homogeneous populations of transfected cells, all containing RCR replicons and expressed sequences, with the need for little or no biochemical selection procedures. This sustained replication in original transfected cells and resulting daughter cells will allow for long term expression experiments and novel application not currently available with standard plasmid vectors or other virus-based vector systems. Due to the basic aspects of the host replication system that RCR replicons require, the replicons will have virtually unlimited host range with regards to cellular replication cycles. These replicons express very few protein products outside of targeted genes or sequences for overexpression and do not perturb host cell metabolism to the same degree that other virus vectors do. All these properties give RCR replicons superior performance and make way for novel utilities not available to other plasmid or virus expression systems. For examples of several utilities, see reduction to practice section.

1. Alternative cellular expression system.

These vectors can be used as an alternative cellular expression vectors and perform superior to plasmid or virus-based vectors based on the following criteria: rapid replication coupled with expression driven by promoter of choice (affecting expression levels or regulation); sustained replication and passive inheritance; unlimited cellular host range; minimal host metabolism perturbation; and, low levels of viral protein accumulation.

2. Enhanced immune response in naked DNA or formulated DNA-based vaccines.

RCR replicons should have sustained replication properties yielding greater levels of substrate for sustained targeted gene expression in transfected cells. The accumulation of targeted immunogen in transfected antigen presenting cells will be greater than standard plasmid vectors. Advantages over virus vectors include: Non-

pathogenic, minimal host perturbation, broad cell host range, no transmission of infection to non-primarily transfected cells due to lack of packaging.

3. Mammalian-cell based genomics using RCR vectors for gene function discovery.

RCR vectors will prove to be excellent gene sequence delivery tools for mammalian genomic approaches. Uses include the expression of homologous or heterologous genes in a library or targeted manner for the detection of gain of function cellular phenotypes and expression of antisense or sense gene fragments for the inhibition of targeted gene expression for assay of loss of function phenotypes.

4. Gene therapy applications

The sustained episomal expression in specific tissues or cells transfected by RCR replicon can allow the delivery of therapeutic or complementing (functional gene copy to complement function of a dysfunctional chromosomal copy) gene products to organisms or cells. The coupling of this activity with the ability of porcine or human brain endothelial cell lines to amplify hemopoetic stem cells without differentiation may prove to be a powerful tool to repopulate a body with new cells containing functional gene copies lacking in native organism. The coupling of these technologies will enable gene therapy to really work. For example, RCR replicon could be designed to express the glucocerebrosidase gene and transfect the hemopoetic stem cells of a patient suffering from Gaucher's disease. Once the transfected stem cells are amplified, they can be re-infused into the patient to engraft in the bone marrow. Once there, the cells will produce a range of hemopoetic cells including Kupffer cells that will be in the liver and responsible for cerebroside lipid degradation. The cells derived from stem cells transfected with the RCR vector will inherit the RCR expression replicon and now express glucocerebrosidase in the liver and now degrade the accumulating lipids that the native system is incapable of doing.

Other properties that are important in RCR vectors to succeed in gene therapy applications: sustained replication, passive episomal replicon inheritance, wide cellular and tissue host range.

5. Unique coupling of RCR vectors with tissue specific gene delivery modalities.

Packaging of RCR replicon DNA in capsid proteins of viruses with specific cellular tropisms (bound by receptors on specific cell or tissue types) for targeted delivery of replicon to tissues in organisms to maximize correct immune response or therapeutic effect. For example, packaging of RCR replicons in papillomavirus virus-like particles (VLPs) would give the replicon a mucosal targeting tropism at the receptor binding step and inherent replication properties of RCR replicons will allow them to amplify and express sequences in mucosal cells. This approach will allow gene delivery for therapeutic end or immunogen delivery for generation of immune response in mucosal tissues (e.g. applications for papilloma, HIV, Herpes virus, Hepatitis B, microbial agents vaccine purposes). Likewise, integration of RCR replicon DNA into adenovirus VLPs would grant pleural tropisms for delivery of genes to advance therapeutic treatment of cystic fibrosis or other diseases. For example, following treatment of patients with DNAse, patients could receive regular treatments with VLPs containing RCR replicons containing therapeutic genes (DNAse gene) or complimenting gene (correct copies of genes causing the disease). In this manner, transient doses of replicons can transfect tissues and deliver therapeutic genes to reverse disease progression. With pleural cell shedding, new transfections with RCR replicons will be necessary to maintain the functional state of pleural tissue.

6. Whole animal genomics or gene therapy

Due to persistent replication and expression and passive inheritance of replicons in daughter cells, it should be possible to transfect pluri-potent cell lineages with replicons containing targeted genes and expect resulting daughters cells and subsequent differentiated cells (or tissues derived from them) to maintain long term expression of the gene of interest. This would allow one to transfect CD34+, CD38- hemopoetic stem cells with RCR vectors in vitro, incubation cells with PMVC or HUBEC cell lines, capable of initiating stem cell cycling and division without inducing differentiation, and then re-infuse transfected cells (now expressing novel gene product) into adult animals. All derived cells from the transfected stem cells will continue to express this novel gene function. This would allow the activities of genes to be ascertained at the entire organismal level and in the context of a variety of cell types.

Conversely, RCR vectors containing homologous or heterologous genes could be delivered to embryos of animals and the organism developing from the transfected embryo would be at least chimeric for the RCR encoded gene function or possibly homogeneous for its expression. This tool could be applied to determine gene function in the context of developing or adult organisms. Alternatively, this could be the ultimate gene therapy tool for complimenting chromosomal defects in organisms, such that the derived organism would continue to have a chromosomal defect in a gene, but it would be complemented by the persistent, episomal gene copy in the RCR vector.

7. Gene therapy through targeted gene repair.

It should be possible to perform directed mutagenesis of a DNA sequence encoded by the host chromosomal genome by encouraging homologous recombination or directed point mutation of specific host DNA sequences homologous to DNA sequences carried on the RCR replicon. Virus infection may induce all necessary factors that are involved in DNA recombination. This coupled with the generation of high levels of homologous recombination substrates: single-stranded DNA during the replication cycle (thought to be more involved in DNA recombination than double stranded DNA) and high copy number of RCR genome in double-stranded DNA form, would be thought to enhance the recombinational frequencies between host chromosome and episomal replicon. For example, when Geminiviruses, containing mutations in the virus coat protein rendering the virus packaging and movement incompetent, are inoculated on transgenic plant hosts containing wild type coat protein ORF, they recombine with transgene locus to recreate a fully functional viral genome at a very high rate (Frischmuth and Stanley, 1998).

Examples

The following examples further illustrate the present invention. These examples are intended merely to be illustrative of the present invention and are not to be considered as limiting.

EXAMPLE 1

Construction of plasmids:

We constructed several plasmids to test whether RCR plasmids carrying the Rep gene and origin of replication could replicate in different mammalian cell types.

Cloning of the genome of PCV

We designed PCR primers to amplify parts of the genome of PCV from the strain present in PK-15 cells (Mehan *et al.*, 1997; Genbank accession no. U49186). For amplification of the whole genome of PCV, with one nucleotide mismatch from the published sequence, the following primers were used; nucleotides identical to the published sequence (Genbank accession no. U49186) are underlined:

PK-15SwaA:
TTTATTTAAATGGAGCCACAGCTGG (SEQ ID NO:3)

PK-15SwaB:
TTTATTTAA-TACCCACACCAATGTCG (SEQ ID NO:4)

In PK-15SwaB, an A has been deleted at position 9 relative to the homologous sequence in the published PCV sequence.

A 1.8-kb fragment was amplified with these primers (not shown), with total nucleic acid (100 ng) isolated from PK-15 cells used as the template for PCR. The PCR fragment was cloned into the pCRblunt-II TOPO vector, according to the manufacturer's instructions (Invitrogen Corp.). A clone containing the correct-sized insert was named construct 1 (Figure 1).

Construct 1 (deposited to ATCC on February 16, 2000, accession no. PTA-1351) thus contained the whole PCV genome cloned into the Invitrogen cloning vector pCR®-Blunt II-TOPO® (Invitrogen Corp.). This construct contained the PCV rep gene under the transcriptional control of its own promoter, and has the putative coat protein inactivated by insertion of the bacterial cloning vector.

For cloning of the PCV genome and expression of its Rep gene under the control of the cytomegalovirus immediate-early promoter (CMV promoter), the PCV genome was amplified by PCR from total DNA isolated from PK-15 cells, using the

following primers; nucleotides identical to the published sequence of PCV are underlined:

PCVwholerepA

ACCATGCCAAGCAAGAAAAGCGGCCC (SEQ ID NO:5)

PCVwholerepB

TTTTCACTGACGCTGCCGAGGTG (SEQ ID NO:6)

A PCR product of approximately 1.8-kb was obtained after PCR amplification using these primers (not shown). This product was cloned into the vector pCMVScript according to the instructions supplied by the manufacturer (Stratagene, La Jolla, California). Construct 2, Shown in Figure 2, contained the PCV genome cloned into the Stratagene vector, pCMV-Script, such that the Rep gene was placed under the control of the cytomegalovirus immediate-early promoter (CMV promoter), with the PCV rep transcription termination and polyadenylation signal and origin of replication sequences upstream. This construct also contained a neomycin/G418 resistance gene with simian virus 40 early promoter (SV40 promoter) and origin of replication sequences, and thus should replicate episomally in COS-7 cells that have an integrated SV40 Large T antigen-expressing gene. The SV40 origin of replication will not, however, be functional in other cell types.

Construct 4 contains the PCV genome amplified with primers PK-15SwaA and PK-15SwaB and cloned into the pCMVScript vector according to the instructions supplied by the manufacturer (Stratagene). This construct therefore contains the PCV Rep gene under the control of its own promoter in a vector which carries an SV40 origin of replication and a selectable marker gene (G418 resistance).

Construct 6 is the Invitrogen pTracerSV40 (Figure 3), which expresses a GFP-zeocin resistance gene fusion, useful because one can evaluate the success of transfection experiments by visualization of green fluorescent protein expression.

Construct 7 (deposited in ATCC on February 16, 2000, accession no. PTA-1352) was derived by deleting the SV40 promoter and origin of replication sequences from pTracer SV40 (Figure 3). The *Nsi*I-*Not*I fragment from construct 1 (Figure 1) was then cloned into the vector. This construct therefore contains the PCV Rep gene under the control of its own promoter, together with the PCV origin of replication sequences, in the context of a vector that contains a selectable and screenable marker gene (GFP-zeocin resistance), but which cannot replicate in COS-7 cells because the SV40 origin of replication sequences have been deleted.

Transfection Experiments with PCV replicons

The RCR vectors described herein may be introduced into eukaryotic cells by one of many different protocols that are available for direct transfer of DNA into cells, including, but not limited to: electroporation, cationic lipid-mediated transfection, calcium phophate transfection, *Agrobacterium*-mediated transfection, microprojectile bombardment, polyethylene glycol-mediated transfection. Several methods that are commonly used for introduction of DNA into mammalian cells are described in detail in "Current Protocols in Molecular Biology" by Ausubel *et al.* (1994-2000). John Wiley and Sons, Inc.

Constructs 1, 2, 4 and 6 were transfected into *Cercopithecus aethiops* (African Green Monkey) kidney cells, transformed with SV40 (COS-7 cells), according to the protocol supplied by the manufacturer of the transfection reagent (Lipofectamine, manufactured by Life Technologies, Inc.)
of COS 7 cells.

Transfections were done in duplicate, i.e. two plates per construct.

1. In a 35 mm tissue culture plate, ~2 x 10^5 cells were seeded in 2 ml D-MEM (Dulbecco's Modified Eagle Medium) containing 10% FBS (Fetal Bovine Serum) and nonessential amino acids (obtained from the ATCC, or from Life Technologies).
2. The cells were incubated at 37°C in a CO_2 incubator until the cells were 70-80% confluent. This took 18-24 hours.
3. The following solutions were prepared in 12 x 75 mm sterile tubes:
   Solution A: For each transfection, 2 µg DNA (plasmid) diluted in 375 µl serum-free D-MEM (containing nonessential amino acids).

Solution B: For each transfection, 6 µl LIPOFECTAMINE Reagent was diluted in 375 µl serum-free D-MEM.

4. The two solutions were combined, mixed gently, and incubated at room temperature for 30 min.
5. The cells were washed once with 2 ml serum-free D-MEM.
6. For each transfection, 750 µl serum-free D-MEM was added to each tube containing the lipid-DNA complexes. After gentle mixing, the diluted complex solution was added onto the washed cells.
7. The cells were incubated for 5 h at 37°C in a CO_2 incubator.
8. 1.5 ml D-MEM with 20% FBS was added without removing the transfection mixture.
9. The medium was replaced at 18-24 h following start of transfection.

Cells were harvested at 2 and 4 days post-transfection. Cells were scraped from the plates and pelleted by centrifugation in 1.5 ml microcentrifuge tubes. Pellets from the duplicate transfection experiments were pooled. We isolated total nucleic acids from these cells, at two and four days post-transfection using the QIAamp DNA Mini Kit, according to the manufacturer's instructions (Qiagen). Two and a half micrograms of total nucleic acids from each sample was digested with 20 units of *Dpn*I, which cuts only *dam*-methylated DNA, i.e. the input plasmid DNA, at sequence GA*TC, where the A* is methylated (Sambrook *et al.*, 1989). The restricted DNA was run on a 1% TAE agarose gel, stained with ethidium bromide. The DNA was transferred to a nylon membrane (Roche Molecular Biochemicals) by the standard alkaline capillary transfer Southern blot protocol (Sambrook *et al.*, 1989). The RCR replicon DNAs were detected by Southern hybridization with a probe made from Construct 1, nonradioactively labeled with Digoxygenin by the random priming method, according to the protocol supplied by the manufacturer (Roche Molecular Biochemicals).

Figure 5 shows the results of the Southern Hybridization experiment. The probe DNA contains sequences (the ColE1 origin of replication) in common with all of the input plasmids, and should therefore hybridize with all replicating, and input plasmid DNAs. Digested, low molecular weight *Dpn* I-digested fragments of the input DNAs

may be visible (less than 1.0-kb, indicated in Figure 4); all replicating DNAs will remain undigested. All plasmids with SV40 ori sequences (constructs 2, 4, and 6) replicated in the COS-7 cells, as expected. Construct 1 (lanes 1 and 2) also appeared to be replicating in the COS-7 cells, indicating that the PCV RCR replicon was functioning, and replicating the linked non-viral DNA sequences. This shows that the PCV-derived RCR replicon can replicate in African Green Monkey kidney cells.

Constructs 2, 4, 6 and 7 were transfected into Chinese Hamster Ovary (CHO) cells. Cells transfected with constructs 6 and 7 exhibited green fluorescence, indicating expression of the GFP fusion protein. Total DNA was isolated from these cells at 2 and 4 days post-transfection. Southern blot analysis showed that constructs 2, 4 and 7, which contain PCV Rep and origin of replication sequences were all replicating in the transfected cells, whereas construct 6, an SV40 replicon, was not replicating. This shows that PCV RCR replicons can replicate, and express genes linked to the replicon, in Chinese Hamster Ovary (CHO) cells.

Constructs 1, 6 and 7 were transfected into COS-7 cells. The transfections were performed according to the methods suggested by the manufacturer of the transfection reagent (Effectene, from Qiagen). We used 1 μg of DNA, 8 μl of enhancer and 25 μl of Effectene per transfection.

Analysis

At one day post-transfection, cells transfected with construct 7 (PCV RCR plasmid) were clearly expressing the GFP-zeocin fusion gene, but cells transfected with construct 6 (with functional SV40 origin of replication sequences) were not. Thus, the PCV replicon expresses linked genes at a higher level, earlier than the cognate SV40 replicon. Constructs 1, 6 and 7 were also transfected into CHO cells, with similar results one day post-transfection.

In another experiment to evaluate GFP gene expression after transfection of CHO-K1 cells, we compared timing and relative intensity of GFP fluorescence after transfection of cells with constructs 6 (non-replicating, with no PCV sequences) and 7 (a PCV-derived construct). Cells were transfected in parallel by two different methods: with Effectene (Qiagen) and with a standard calcium phosphate precipitation protocol.

Effectene transfection method

For the effectene transfection method, one microgram of plasmid DNA was mixed with DNA condensation buffer (Buffer EC), to a total volume of 150 µl. Eight microlitres of Enhancer were added, and the DNA solution was mixed by vortexing for one second. The DNA mixture was incubated at room temperature for 5 minutes. Effectene transfection reagent (25 µl) was added to the DNA-enhancer mixture, and mixed by pipetting up and down five times. The samples were incubated at room temperature to allow complex formation.

While complex formation was occurring, the growth medium was gently aspirated from the plates, and the cells were washed once with phosphate buffered saline (PBS). Four milliliters of fresh growth medium was then added to the cells.

One milliliter of growth medium was added to the reaction tube containing the transfection complexes; the solution was then mixed and immediately added drop-wise onto the cells in 60-mm dishes. The dish was gently swirled to ensure uniform distribution of the complexes. The cells with transfection complexes were incubated at 37°C and 5% CO_2 to allow for gene expression.

The expression of GFP in transfected cells was observed at three and seven days post-transfection. The results from observation of cells transfected by the Effectene method are tabulated below (Table 1).

Construct 3: non-replicating plasmid DNA, no GFP gene (DNA control)

Construct 6: non-replicating plasmid DNA, GFP-zeocin fusion gene, should express GFP in transfected cells.

Construct 7: plasmid DNA with PCV replicon and the same GFP-zeocin fusion gene Construct 6: may be capable of replication.

Table 1

Dish #	Construct	Day 3 Observations	Day 7 Observations
1	3	No GFP, cells look healthy	No GFP, cells growing well
2	6	4 to 5% GFP +ve, low level expression	Small number of GFP +ve cells
3	7	10% GFP +ve, low to moderate expression	Very many GFP +ve cells, both dim and bright

5	No DNA	No GFP; cells growing well	No GFP

EXAMPLE 2

We constructed plasmid pCI-PCV1-Luc (FIG. 7) that contained the Firefly luciferase gene as a reporter gene to obtain quantitative data on the expression-enhancing effect of PCV-based gene vectors (the nucleotide sequence is depicted in SEQ ID NO:7).

Vector construction

To measure the effect of the PCV1 genome on the stability and expression of a reporter gene, the PCV1 genome was amplified by PCR using the primer PCV BglStu (AAAGATCTAGGCCTGTGTGCTCGACATTG) (SEQ ID NO:8) and PCVBamStu (AAGGATCCAGGCCTCGGCTATGCGCTCC) (SEQ ID NO:9). These primers amplified the circular PCV1 genome from nucleotide 1130, the point of a unique StuI site, and also introduced a BglII and BamHI restriction site at the 5' and 3' end respectively. The PCR product was than digested with the restriction enzymes BglII and BamHI and introduced into the commercially available plasmids pGL3 (Genbank accession no. U47298) to generate pCI-PCV1-Luc (Figure 7) and into the pCI mammalian expression vector (Genbank accession no. U47119) linearized at nucleotide 1 by a BglII digestion to generate pGL3-PCV1-Luc (Figure 9). The Luciferase gene was already contained in the pGL3 construct and was added as an EcoRI-NheI fragment into the pCI and pCI-PCV1 construct. The DNA sequence of pCI-PCV1-Luc with the inserted luciferase gene is shown in Figure 8.

After sequence confirmation, the DNA from each construct was purified using a DNA maxiprep kit (Qiagen) and used for cell transfection experiments.

Cell transfection

CHO-K1 cells (Chinese hamster ovary epithelial cells ATCC no. CCL-61) were maintained in F12K HAM media (Gibco BRL) supplemented with 10% Fetal Bovine Sera. 24 hours before transfection, cells were trypsinized and dispensed into a 24 well plate at 1 x 10^4 cells/well.

Before transfection, cells were washed with PBS and fresh media was added. Transfection was performed with the Effected kit (Qiagen). For each construct,

250ng of DNA/well was used. A ratio of 8 µl of enhencer and 25 µl of effectene reagent per µg of DNA was used. For each construct duplicate experiments were performed. 24 hours after transfection the media was changed.

To assess the Luciferase activity, the cells were lysed at different time post-transfection by a 15 minutes room temperature incubation of the cells with the passive lysis buffer included in the Luciferase assay kit (Promega). The Luciferase expression units were measured with a Luminometer (TD-20/20, Turner designs) with an integration period of 10 sec. The results are shown as an average of the total Firefly luciferase (F. Luc) units measured.

The results show that substantially higher levels of luciferase expression are seen in cells transfected with a luciferase expression cassette linked to a PCV1 replicon. This phenomenon was independent of the promoter used to drive expression of the luciferase gene: that is, the cytomegalovirus immediate-early promoter (See Figure 10A, compare pCI (no PCV sequences) with pCI-PCV1 (PCV sequences linked to the expression cassette) or the simian virus 40 promoter (see Figure 10B, compare pGL3, with no PCV sequences linked to the SV40-luciferase expression cassette, vs pGL3-PCV1, with PCV sequences linked to the SV40-luciferase expression cassette).

EXAMPLE 3

We constructed plasmids pCI-Luc and pCI-β gal (FIG. 11A) and pCI-PCV1-Luc (FIG. 7) and pCI-PCV1-β gal (FIG. 11B) to express either luciferase (F. Luc) or beta-galactosidase (β gal) *in vivo*, and quantitated IgG upregulation in injected mice.

Vector construction

To assess the immunizing ability of the rolling circle replicon vectors against foreign antigens, we constructed plasmids pCI-PCV1-β gal and pCI-Luc and pCI-β gal. Plasmid pCI-PCV1-Luc was described previously (FIG. 7; SEQ ID NO: 7).

pCI-PCV1-β gal was constructed essentially as for pCI-PCV1-Luc as mentioned above in Example 2. Briefly, the PCV1 genome was amplified as above by PCR. The PCR product was then digested with the restriction enzymes BglII and BamHI and introduced into the commercially available pCI mammalian expression vector (Genbank accession no. U47119) linearized at nucleotide 1 by a BglII

digestion. The luciferase gene was added as an EcoRI-NheI fragment into the pCI construct (FIG. 11A). The β-galactosidase gene was added as an insert fragment in a recombinase reaction to converted Gateway (LifeTechnologies/Invitrogen) pCI and pCI-PCV1 constructs to make pCI-β gal (FIG. 11A) and pCI-PCV1-β gal (FIG. 11B), respectively.

After sequence confirmation, the DNA from each construct was purified using an endotoxin free DNA maxiprep kit (Qiagen). The eluted DNA was ethanol precipitated and resuspended in normal saline.

DNA immunization

100 micrograms of DNA was injected intramuscularly in the thigh of each of five mice per group, once per week for three weeks. Sera were collected one week after each immunization, pooled, and analyzed for antibody titre using ELISA.

Antibody titre measurements

Purified antigen (either firefly luciferase or beta-galactosidase, dependent upon the expression vector utilized) was bound to the surface of polystyrene ELISA plates, and washed extensively with TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20) to remove any excess protein. The plates were blocked with blocking buffer (2% bovine serum albumin in 50 mM Tris, pH 8.0, 0.02% Tween-20 (a non-ionic detergent compound)) and incubated with serial dilutions of pooled sera from mice injected with various treatments. The plates were then washed extensively with TBST and probed with peroxidase-conjugated anti-mouse IgG antibody (Southern Biotech). The plates were developed with the addition of o-phenylenediamine in citrate buffer containing 0.012% H_2O_2 and the absorbance measured at 405 nm using a plate spectrophotometer (Molecular Devices).

Antibody isotype switching measurements

Purified antigen (either firefly luciferase or beta-galactosidase, dependent upon the expression vector utilized) was bound to the surface of polystyrene ELISA plates as above, and washed extensively with TBST to remove any excess protein. The plates were blocked with blocking buffer (2% bovine serum albumin in 50 mM Tris, 0.02% Tween-20) and incubated with serial dilutions of pooled sera from mice

injected with various treatments. The plates were washed extensively with TBST and probed with peroxidase-conjugated anti-mouse antibodies which specifically recognize either IgG1, IgG2a, or IgG2b (Southern Biotech). The plates were developed with the addition of o-phenylenediamine in citrate buffer containing 0.012% H_2O_2 and the absorbance measured at 405 nm using a plate spectrophotometer (Molecular Devices).

Results

FIGS. 12A and 12B show that substantially higher levels of IgG antibody induction can be seen in mice injected with immunogenic protein insert linked to a rolling circle replicon cassette (pcv) versus immunogenic protein inserted into a non-rolling circle replicon mammalian expression vector (pci). Levels of IgG antibody through successive bleeds (b1 = week 1, b2 = week 2, b3 = week 3 and b4 = week 4) were higher for both luciferase and β-galactosidase expression cassettes linked to a PCV cassette, as compared to non-rolling circle replicon linked vectors (pci) or injection with saline alone (pbs), vector without insert (vector), purified luciferase (luc) or β-galactosidase (bGal). This induction was independent of the promoter used to drive expression of the heterologous genes, that is, the cytomegalovirus immediate-early promoter (see FIGS. 12A and 12B, compare pci to pcv antibody induction).

This IgG antibody induction by PCV-linked expression vectors was selective for specific IgG isotypes, indicating the activation of a long-term versus short-term immunogenic response. FIGS. 13A and 13B show that mice injected with PCV-linked expression cassettes have sustained levels of IgG2a and IgG2b induction, versus IgG1. This indicates a T-cell mediated antibody response, more indicative of a long-term memory immunogenic reaction.

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Although the invention has been described with reference to the presently preferred embodiments, it should be understood that various modifications could be made without departing from the spirit of the invention.